	L #	Hits	Search Text	DBs	Time Stamp
1	L1	355	sulfolobus or acidocaldarius	USPAT; US-PGPUB	2002/06/05 15:10
2	L2	3835	trehalose	USPAT; US-PGPUB	2002/06/05 15:11
3	L4	110	non adj reducing adj saccharide$1	USPAT; US-PGPUB	2002/06/05 15:11
4	L6	3856	$trehalose	USPAT; US-PGPUB	2002/06/05 15:12
5	L7	31	1 and 6	USPAT; US-PGPUB	2002/06/05 15:12
6	L3	31	1 and 2	USPAT; US-PGPUB	2002/06/05 15:13
7	L5	19	1 and 4	USPAT; US-PGPUB	2002/06/05 15:29
8	L8	112	(2 or 4) near6 (synthes$8 or produc$8 or form$6) near6 enzym$8	USPAT; US-PGPUB	2002/06/05 15:39

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	355	sulfolobus or acidocaldarius	USPAT; US-PGPUB	2002/06/05 15:10
2	L2	3835	trehalose	USPAT; US-PGPUB	2002/06/05 15:11
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5	L6	3856	$trehalose	USPAT; US-PGPUB	2002/06/05 15:12
6	L7	31	1 and 6	USPAT; US-PGPUB	2002/06/05 15:12
7	L3	31	1 and 2	USPAT; US-PGPUB	2002/06/05 15:13

US-PAT-NO: 6391595
DOCUMENT-IDENTIFIER: US 6391595 B1
TITLE: Transferase and amylase, process for producing the enzymes, use thereof, and gene coding for the same
DATE-ISSUED: May 21, 2002
US-CL-CURRENT: 435/100,435/183 ,435/194 ,435/200 ,435/91.53 ,435/97

APPL-NO: 9/ 298924
DATE FILED: April 26, 1999
PARENT-CASE:
This application is a Divisional of application Ser. No. 08/750,569, filed Feb. 24, 1997, which is a national stage of PCT/JP95/01189 filed Jun. 14, 1995.

FOREIGN-APPL-PRIORITY-DATA:
FOREIGN-PRIORITY-APPL-NO: JP 6-133354
FOREIGN-PRIORITY-APPL-DATE: June 15, 1994

US-PAT-NO: 6346394
DOCUMENT-IDENTIFIER: US 6346394 B1
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: February 12, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/69.1,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.2 ,435/71.1 ,435/71.2 ,435/97 ,536/23.1 ,536/23.2 ,536/23.7
ABSTRACT:

Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of trehalose on an industrial scale and in a satisfactorily-high yield.

17 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

DATE FILED: April 6, 1998

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TTL:
Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide

ABPL:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of trehalose on an industrial scale and in a satisfactorily-high yield.

BSPR:
The present invention relates to a recombinant thermostable enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:
Trehalose is a disaccharide which consists of 2 glucose molecules that are linked together with their reducing groups, and, naturally, it is present in fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, trehalose does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can advantageously sweeten food products without fear of causing unsatisfactory coloration and deterioration. Trehalose, however, could not have been readily prepared in a desired amount by conventional production methods, so that it has not scarcely been used for sweetening food products.

BSPR:
Conventional production methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other using a multi-enzymatic system where several enzymes are allowed to act on saccharides. The former, as disclosed in Japanese Patent Laid-Open No.154,485/75, is a method which comprises growing microorganisms such as bacteria and yeasts in nutrient culture media, and collecting trehalose mainly from the proliferated cells. The latter, as disclosed in Japanese Patent Laid-Open No.216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and trehalose-phosphorylases to act on maltose, and recovering the formed trehalose from the reaction system. The former facilitates the growth of microorganisms, but has a demerit that the content in the microorganisms is at most 15 w/w %, on a dry solid basis (d.s.b.). Although the latter can readily separate trehalose, it is theoretically difficult to increase the trehalose yield by allowing such phosphorylases to act on substrates at a considerably-high concentration because the enzymatic reaction in itself is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

BSPR:
In view of the foregoing, the present inventors energetically screened enzymes which form saccharides having a trehalose structure from amylaceous saccharides, and have found that microorganisms such as those of the genera Rhizobium and Arthrobacter produce an absolutely novel enzyme which forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. They disclosed such an enzyme in Japanese Patent Application No.349,216/93. At almost the same time, they also found that these non-reducing saccharides are nearly quantitatively hydrolyzed into trehalose and glucose and/or maltooligosaccharides by other enzymes produced from the same microorganisms of the genera Rhizobium and Arthrobacter.

BSPR:
It was found that the enzymes produced from the aforesaid microorganisms have an optimum temperature of about 40.degree. C., and have some difficulties in their thermostability when actually used to produce trehalose. It is recognized in this field that a recommendable temperature in the saccharification reaction of starch or amylaceous saccharides is one which exceeds 55.degree. C. because bacterial contamination will occur at a temperature of 55.degree. C. or lower and decreasing the pH of the reaction

mixtures and inactivating the enzymes used. Thus, a relatively-large amount of substrates remain intact. While the use of enzymes with a poor thermostability, a great care should be taken to control the pH, and, when the pH level lowers to an extremely low level, alkalis should be added to reaction mixtures to increase the pH level as quickly as possible.

BSPR:
In view of the foregoing, the present inventors screened thermostable enzyme with a satisfactory activity and have found that enzymes produced from microorganisms of the genus Sulfolobus including Sulfolobus acidocaldarius (ATCC 33909) are not substantially inactivated even when incubated at a temperature exceeding 55.degree. C., and they efficiently release trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. These microorganisms, however, are not sufficient in the enzyme productivity, and this requires a relatively-large scale culture to industrially produce trehalose from those non-reducing saccharides.

BSPR:
It is an object of the present invention to provide a recombinant thermostable enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3 by using the recombinant DNA technology.

BSPR:
It is another object of the present invention to provide an enzymatic conversion method for releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:
The sixth object of the present invention is attained by an enzymatic conversion method of non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, which contains a step of allowing the recombinant thermostable enzyme to act on the non-reducing saccharides to release trehalose.

BSPW:
Releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3;

DRPR:
FIG. 1 is a figure of the optimum temperature of a thermostable enzyme produced from Sulfolobus acidocaldarius (ATCC 33909).

DRPR:
FIG. 2 is a figure of the optimum pH of a thermostable enzyme produced from Sulfolobus acidocaldarius (ATCC 33909).

DRPR:
FIG. 3 is a figure of the thermostability of a thermostable enzyme produced from Sulfolobus acidocaldarius (ATCC 33909).

DRPR:
FIG. 4 is a figure of the pH stability of a thermostable enzyme produced from Sulfolobus acidocaldarius (ATCC 33909).

DEPR:
The recombinant thermostable enzyme according to the present invention releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3 without substantial inactivation even when allowed to react at a temperature exceeding 55.degree. C.

DEPR:
According to the present enzymatic conversion method converts non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3 into saccharide compositions containing trehalose and glucose and/or maltooligosaccharides.

DEPR:
The present invention has been made based on the finding of a novel enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. Such an enzyme is obtainable from cultures of microorganisms of the species Sulfolobus acidocaldarius (ATCC 33909). The present inventors isolated such an enzyme by using in combination a various purification methods comprising column chromatography as a main technique, studied their properties and features, and revealed that the reality is a polypeptide with the following physicochemical properties:

DEPR:
The followings are the explanations of the experiments conducted to reveal the physicochemical properties of a thermostable enzyme produced from Sulfolobus acidocaldarius (ATCC 33909):

DEPR:
Into 500-ml flasks were poured 100 ml aliquots of a liquid culture medium containing 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min. After cooling the flasks a seed culture of Sulfolobus acidocaldarius (ATCC 33909) was inoculated into each liquid culture medium in each flask, followed by the incubation at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was poured into a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the first seed culture into the sterilized liquid culture medium in the fermenter, and culturing the microorganisms at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was poured into a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the second seed culture into the sterilized liquid culture medium, and culturing the microorganisms at 75.degree. C. for 42 hours under an aeration condition of

100 L/min.

DEPR:
The results in Table 1 show that the purified enzyme nearly quantitatively releases trehalose and glucose or maltooligosaccharides from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, but does not substantially act on maltooligosaccharides having a degree of glucose polymerization of at least 3. These facts indicate that the purified enzyme specifically acts on non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, and specifically hydrolyzes the glycosidic linkages between trehalose- and glycosyl-residues. Such an enzyme has not been reported and it can be estimated to have a novel enzymatic pathway.

DEPR:
A chromosomal DNA of Sulfolobus acidocaldarius (ATCC 33909) was screened by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequences in SEQ ID NOs:3 and 4, and this yielded a DNA fragment having a base sequence (SEQ ID NO:2.) from the 5'-terminus consisting of about 1,700 base pairs. The base sequence of the thermostable enzyme was decoded and revealing that it consists of 556 amino acids and has a partial amino acid sequence from the N-terminal in SEQ ID NO:1.

DEPR:
To 500-ml flasks were placed about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to a pH 3.0 by the addition of sulfate. A seed culture of Sulfolobus acidocaldarius (ATCC 33909) was inoculated into each flask, incubated at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a seed culture. About 5 L of a fresh preparation of the same liquid nutrient culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to a pH 3.0, and inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min.

DEPR:
As a control, a seed culture of Escherichia coli XLI-Blue strain or Sulfolobus acidocaldarius (ATCC 33909) was inoculated into a fresh preparation of the same liquid culture medium but free of ampicillin. In the case of culturing Sulfolobus acidocaldarius (ATCC 33909), it was cultured and treated similarly as above except that the initial pH of the nutrient culture medium and the culturing temperature were respectively set to 3.0 and 75.degree. C. Assaying the resultant enzymatic activity, one L culture of Sulfolobus acidocaldarius (ATCC 33909) yielded about 2 units of the thermostable enzyme, and the yield was significantly lower than that of transformant SU18. Escherichia coli XLI-Blue strain used as a host did not form the thermostable enzyme.

DEPR:
Thereafter, the recombinant thermostable enzyme produced by the transformant SU18 was purified similarly as in Experiments 1 and 2 and examined for

properties and features and revealing that it has substantially the same physicochemical properties of the thermostable enzyme from Sulfolobus acidocaldarius (ATCC 33909) because (i) the recombinant thermostable enzyme has a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and an isoelectric point of about 5.6-6.6 on isoelectrophoresis, and (ii) it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These results indicate that the present thermostable enzyme can be prepared by the recombinant DNA technology with a significantly improved yield.

DEPR:
Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of about 1,700 base pairs in SEQ ID NO:5. An amino acid sequence that could be estimated from the base sequence was in SEQ ID NO:5, and it was compared with the partial amino acid sequences in SEQ ID NOs:3 and 4, and revealing that the partial amino acid sequence in SEQ ID NO:3 corresponded to that positioning from 1 to 30 in SEQ ID NO:5, and that in SEQ ID NO:4 corresponded to that positioning from 301 to 319 in SEQ ID NO:5. These results indicate that the present recombinant thermostable enzyme has the amino acid sequence from the N-terminal in SEQ ID NO:1, and, in the case of the DNA derived from Sulfolobus acidocaldarius (ATCC 33909), the amino acid sequence is encoded by the base sequence from the 5'-terminus in SEQ ID NO:2.

DEPR:
As is explained in the above, the thermostable enzyme, which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, was found as a result of the present inventors' long-term research. The thermostable enzyme has distinct physicochemical properties from those of other conventional enzymes. The present invention is to produce the thermostable enzyme by using the recombinant DNA technology. The present recombinant thermostable enzyme, its preparation and uses will be explained in detail with reference to the later described Examples.

DEPR:
The recombinant thermostable enzyme as referred to in the present invention means thermostable enzymes in general which are preparable by the recombinant DNA technology and capable of releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. Generally, the recombinant thermostable enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence from the N-terminal as shown in SEQ ID NO:1, and homologous ones to it can be mentioned. Variants having amino acid sequences homologous to the one in SEQ ID NO:1 can be obtained by replacing one or more bases in SEQ ID NO:1 with other bases without substantially alternating the inherent physicochemical properties. Although even when used the same DNA and it also depends on hosts into which the DNA is introduced, the ingredients and components of nutrient culture media for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have the inherent physicochemical properties but defect one or more amino acids in SEQ ID NO:1, or those which have one or more amino acids added newly to the N-terminal after the DNA expression as the result of the modification of

intracellular enzymes of the hosts. Such variants can be used in the present invention as long as they have the desired physicochemical properties.

DEPR:
The DNA usable in the present invention includes those are derived from natural resources and those which are artificially synthesized as long as they have the aforesaid base sequences. The natural resources for the DNA according to the present invention are, for example, microorganisms of the genus Sulfolobus such as Sulfolobus acidocaldarius (ATCC 33909), and from which genes containing the present DNA can be obtained. The aforementioned microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or .beta.-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, when treated the cells with an ultrasonic disintegrator, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or with freezing and thawing method. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment generally used in this field. To artificially synthesize the present DNA, it can be chemically synthesized by using the base sequence in SEQ ID NO:2, or can be obtained in a plasmid form by inserting a DNA, which encodes the amino acid sequence in SEQ ID NO:1, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the objective DNA from the cells.

DEPR:
The recombinant DNA thus obtained can be introduced into appropriate host microorganisms including Escherichia coli and those of the genus Bacillus as well as actinomyces and yeasts. In the case of using Escherichia coli as a host, the DNA can be introduced thereinto by culturing the host in the presence of the recombinant DNA and calcium ion, while in the case of using a microorganism of the genus Bacillus as a host the competent cell method and the colony hybridization method can be used. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, and selecting the objective transformants which release trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3.

DEPR:
The transformants thus obtained intra- and extra-cellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid culture media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with small amounts of amino acids and vitamins can be used in the invention. Examples of the carbon sources are saccharides such as unprocessed starch, starch hydrolysate, glucose, fructose, sucrose and trehalose. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia and salts

thereof, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor, and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 20-65.degree. C. and a pH of 2-9 for about 1-6 days under aerobic conditions by the aeration-agitation method. Such cultures can be used intact as a crude enzyme, and, usually, cells in the cultures may be disrupted prior to use with ultrasonic and/or cell-wall lysis enzymes, followed by separating the thermostable enzyme from intact cells and cell debris by filtration and/or centrifugation and purifying the enzyme. The methods to purify the enzyme include conventional ones in general. From cultures intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

DEPR:
As is described above, the recombinant thermostable enzyme according to the present invention has a specific feature of releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3 even when allowed to act on them at a temperature exceeding 55.degree. C. The trehalose thus obtained has a satisfactorily-mild and high-quality sweetness as well as an adequate viscosity and moisture-retaining ability, and, as a great advantageous feature, they can sweeten food products without fear of causing unsatisfactory coloration and deterioration because they have no reducing residue within their molecules. With these features a variety of amylaceous saccharides, which have been put aside because of their reducibilities, can be converted into saccharides which have a satisfactory handleability, usefulness, and no substantial reducibility or extremely-reduced reducibility.

DEPR:
Explaining now the conversion method in more detail, non-reducing saccharides having a trehalose structure and a degree of glucose polymerization of at least 3 such as .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose. These non-reducing saccharides can be obtained by allowing a non-reducing saccharide-forming enzyme as disclosed in Japanese Patent Application No.349,216/93, applied by the present applicant and Japanese Patent Application Serial No.10046601, titled "Thermostable non-reducing saccharide-forming enzyme, its preparation and uses", applied by the same applicant on Jun. 24, 1994, to act on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 which are prepared by treating starch or amylaceous saccharides such as amylopectin and amylose with acids and/or amylases. These reducing saccharides usable as a substrate for the non-reducing saccharide-forming enzyme usually contain one or more maltooligosaccharides having a degree of glucose polymerization of at least 3, for example, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. As is described in "Handbook of Amylases and Related Enzymes", 1st edition (1988), edited by The Amylase Research Society of Japan, published by Pergamon Press plc, Oxford, England, .alpha.-amylase, maltotetraose-forming amylase, maltopentaose-forming amylase and maltohexaose-forming amylase are especially useful to prepare the reducing amylaceous saccharides used in the

present invention, and, the use of any one of these amylases facilitates the production of mixtures of amylaceous saccharides rich in reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 in a considerably-high yield. If necessary, the combination use of the amylases and starch debranching enzymes such as pullulanase and isoamylase can increase the yield of the reducing amylaceous saccharides used as the substrate for the present recombinant thermostable enzyme. Non-reducing saccharides can be obtained in a desired amount by coexisting such a non-reducing saccharide-forming enzyme in aqueous solutions containing one or more reducing amylaceous saccharides up to 50 w/w %, and, usually, incubating the mixture solution at a temperature of 40-85.degree. C. and a pH of about 4-8 until the non-reducing saccharides are produced.

DEPR:
In the enzymatic conversion method according to the present invention, the present recombinant thermostable enzyme is generally allowed to coexist in an aqueous solution containing one or more of the above non-reducing saccharides as a substrate, followed by the enzymatic reaction at a prescribed temperature and pH until a desired amount of trehalose is formed. Although the enzymatic reaction proceeds even at a concentration of about 0.1 w/w %, d.s.b., of a substrate, a concentration of 2 w/w % or higher, d.s.b., preferably, in the range of 5-50 w/w %, d.s.b., of a substrate can be satisfactorily used when used the present conversion method in an industrial-scale production. The temperature and pH used in the enzymatic reaction are set to within the range of which does not inactivate the recombinant thermostable enzyme and allows the enzyme to effectively act on substrates, i.e. a temperature of higher than 55.degree. C. but not higher than 85.degree. C., preferably, a temperature in the range of about 56-70.degree. C., and a pH of 4-7, preferably, a pH in the range of about 5-6. The amount and reaction time suitable for the present recombinant thermostable enzyme are chosen depending on the enzymatic reaction condition. Thus, the present recombinant thermostable enzyme effectively converts non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3 into trehalose and glucose and/or maltooligosaccharides, e.g. the conversion rate increases up to about 99% when the enzyme acts on .alpha.-maltotriosyltrehalose. When either of amylases is allowed to act on starch hydrolysates in combination with the non-reducing saccharide-forming enzyme and the present thermostable enzyme, non-reducing saccharides are formed along with trehalose and glucose and/or maltooligosaccharides. Thus, saccharide compositions rich in trehalose are efficiently formed in a relatively-high yield.

DEPR:
The trehalose and compositions containing it have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
The purified enzyme was assayed for properties and features by the method in Experiment 2 and revealing that it had a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and a pI of about 5.6-6.6 on isoelectrophoresis, and was not substantially inactivated even when incubated

in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These physicochemical properties were substantially the same as those of the thermostable enzyme from a donor microorganism of Sulfolobus acidocaldarius (ATCC 33909).

DEPR:
To 500-ml flasks were added about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water. The flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled and adjusted its pH to 3.0 by the addition of sulfuric acid. A seed culture of Sulfolobus acidocaldarius (ATCC 33909) was inoculated to the flasks, and cultured at 75.degree. C. for 24 hours under a rotatory shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to pH 3.0, inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min to obtain a second seed culture. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium in a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and cultured under aeration and agitation conditions of 100 L/min for 42 hours.

DEPR:
Fractions with an enzymatic activity eluted at about 0.8 M ammonium sulfate were collected, pooled, dialyzed for 16 hours against 10 mM Tris-HCl buffer (pH 8.5) containing 0.2 M sodium chloride, and centrifuged at 10,000 rpm for 30 min to remove insoluble substances. The resultant supernatant was fed to a column packed with about 350 ml of "TOYOPEARL.RTM. HW-55", a gel for gel chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 0.2 M sodium chloride. Fractions with an enzymatic activity were collected from the eluate, pooled, and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) for 16 hours. The dialyzed solution was centrifuged to remove insoluble substances, and the supernatant was subjected to hydrophobic column chromatography using "MONO Q", a gel for ion-exchange chromatography commercialized by Pharmacia LKB Uppsala, Sweden, followed by feeding to the column with a linear gradient buffer ranging from 0 M to 0.2 M of sodium chloride in 10 mM Tris-HCl buffer (pH 8.5). The fractions eluted at about 0.1 M sodium chloride were collected and pooled for the production of trehalose. The purified non-reducing saccharide-forming enzyme thus obtained had a specific activity of about 81 units/mg protein, and the yield was about 0.24 units per one L of the culture.

DEPR:
The syrup had a relatively-low DE (dextrose equivalent) and contained 71.0 w/w % trehalose, 2.9 w/w % glucosyltrehalose, 1.0 w/w % maltosyltrehalose, 4.9 w/w % glucose, 10.5 w/w % maltose, 8.2 w/w % maltotriose and 1.5 w/w % maltotetraose and higher maltooligosaccharides, d.s.b. The product, having a mild and moderate sweetness as well as an adequate viscosity and moisture-retaining ability, can be satisfactorily used in compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient

and adjuvant.

DEPR:
A syrupy product in Example B-1 was column chromatographed using a strong-acid cation exchange resin to increase the trehalose content. The procedures were as follows: Four jacketed-stainless steel columns, 5.4 cm in diameter and 5 m in length each, were packed to homogeneity with "XT-1016 (Na.sup.+ -form)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, which had been previously suspended in water, and cascaded in series to give a total column length of 20 m. The columns were fed with the syrupy product, adequately diluted with water, in a volume of about 5 v/v % to the resin and at an inner column temperature of 55.degree. C., and fed with 55.degree. C. hot water at an SV (space velocity) 0.13 to elute saccharide components. Fractions rich in trehalose were collected, pooled, concentrated, dried in vacuo and pulverized to obtain a powdery product containing about 97 w/w % trehalose in a yield of about 55 w/w % to the material, d.s.b.

DEPR:
A fraction rich in trehalose obtained by the method in Example B-2 was concentrated into an about 75 w/w % solution which was then transferred to a crystallizer, and crystallized under gently stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was sprayed downward from a nozzle equipped on the upper part of a spraying tower at a pressure of about 150 kg/cm.sup.2 while an about 85.degree. C. hot air was blowing downward from the upper part of the spraying tower, and the formed crystalline powder was collected on a wire-netting conveyer provided on the basement of the drying tower and gradually conveyed out of the spraying tower while an about 45.degree. C. hot air was blowing to the crystalline powder from under the conveyer. The crystalline powder thus obtained was transferred to an ageing tower and aged for 10 hours in a stream of about 40.degree. C. hot air to complete the crystallization and drying. Thus, a powdery hydrous crystalline trehalose was obtained in a yield of about 90 w/w % to the material, d.s.b.

DEPR:
Tapioca starch was dissolved in water into a 36 w/w % suspension which was then admixed with 0.1 w/w % calcium carbonate. The mixture was adjusted to pH 6.0, admixed with 0.2 w/w % of "TERMAMYL 60L", an .alpha.-amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, to starch, d.s.b., and enzymatically reacted at 95.degree. C. for 15 min to gelatinize and liquefy the starch. The mixture was autoclaved at 120.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 58.degree. C., adjusted to pH 5.2, admixed with 2,000 units/g starch, d.s.b., of an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 2.5 units/g starch, d.s.b., of a thermostable enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.s.b, of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 72 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 50.degree. C., admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to

inactivate the remaining enzyme, cooled, filtered, decolored in usual manner with an activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 75.5 w/w % trehalose, d.s.b.

DEPR:
The syrupy product was concentrated into an about 84 w/w % solution which was then transferred to a crystallizer, admixed with about 2 w/w %, d.s.b., of hydrous crystalline trehalose as a seed crystal, and crystallized under gentle stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was distributed to plastic plane vessels, allowed to stand at ambient temperature for 3 days to solidify and age the contents. Thereafter, the formed blocks were removed from the vessels, powdered by a pulverizer to obtain a solid product containing hydrous crystalline trehalose in a yield of about 90 w/w % to the material starch, d.s.b.

DEPR:
Potato starch was suspended in water into a 6 w/w % suspension which was then admixed with 0.01 w/w % "NEO-SPITASE", an .alpha.-amylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, adjusted to pH 6.2, and subjected to an enzymatic reaction at a temperature of 85-90.degree. C. for one hour to gelatinize and liquefy the starch. The mixture was heated at 120.degree. C. for 10 min to inactivate the remaining enzyme, cooled to 60.degree. C., adjusted to pH 5.5, admixed with 500 units/g starch, d.s.b., of "PROMOZYME 200L", a pullulanase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, 3.0 units/g starch, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered, decolored in usual manner with an activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 79.3 w/w % trehalose, d.s.b.

DEPR:
The syrupy product was column chromatographed similarly as in Example B-2 except that "C6000", commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used a strong-acid cation exchange resin in Na.sup.+ -form, followed by collecting a fraction containing about 95 w/w % trehalose, d.s.b. The fraction was concentrated up to about 75 w/w % and crystallized similarly as in Example B-4 to obtain a massecuite in the from of block which was then pulverized to obtain a powdery product containing hydrous crystalline trehalose in a yield of about 70 w/w % to the material starch, d.s.b.

DEPR:
One part by weight of "EX-I", an amylose product commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was dissolved by heating in 15

parts by weight of water, and the solution was adjusted to 65.degree. C. and pH 5.5, admixed with 2.0 units/g amylose, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a) and 6.0 units/g amylose, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-2, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was incubated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and pH 5.0, admixed with 10 units/g amylose, d.s.b., "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and incubated for another 40 hours. The newly formed reaction mixture was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered in usual manner, decolored with an activated charcoal, deionized and purified with an ion exchanger, and concentrated up to give a concentration of about 60 w/w % to obtain a syrupy product containing 82.2 w/w % trehalose, d.s.b.

DEPR:
The syrupy product was similarly as in Example B-5 subjected to column fractionation to obtain a fraction containing 98 w/w % trehalose, d.s.b., which was then concentrated by heating under a reduced pressure up to give a concentration of about 85 w/w %. To the concentrate was added about 2 w/w % anhydrous crystalline trehalose as a seed, followed by mixing the resultant at 120.degree. C. for 5 min under stirring conditions. The resultant mixture was distributed to plastic plain vessels, and crystallized by drying in vacuo at 100.degree. C. Thereafter, products in the form of a block were removed from the vessels, pulverized with a cutter to obtain a solid product, which contained anhydrous crystalline trehalose and had a moisture content of about 0.3 w/w % and a crystallization percentage of about 70 w/w %, in a yield of about 70% to the material amylose, d.s.b.

DEPR:
Anhydrous crystalline trehalose absorbs moisture from anhydrous substances to convert into hydrous crystalline trehalose, and because of this the product rich in such anhydrous crystalline trehalose is useful as a desiccant to dehydrate compositions such as food products, cosmetics and pharmaceuticals, and their materials and intermediates. The product with a mild and high-quality sweetness can be arbitrarily incorporated into compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, diluent and excipient.

DEPR:
As is described above, the present invention is based on the finding of a novel thermostable enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The present invention is to explore a way to produce such a thermostable enzyme in an industrial scale and in a relatively-high efficiency by the recombinant DNA technology. The present conversion method using the recombinant thermostable enzyme readily converts non-reducing amylaceous saccharides, which have a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, into trehalose and glucose and/or maltooligosaccharides without fear of causing bacterial contamination. The trehalose has a mild and high-quality sweetness, and, because it has no reducing residue within the molecule, it can be

advantageously incorporated into compositions in general such as food products, cosmetics and pharmaceuticals without fear of causing unsatisfactory coloration and deterioration. The present recombinant thermostable enzyme is one which has a revealed amino acid sequence, so that it can be used freely in the preparation of trehalose that is premised to be used in food products and pharmaceuticals.

DEPL:
Conversion into Syrupy Product Containing Trehalose

DEPL:
Conversion into Syrupy Product Containing Trehalose

DEPL:
Conversion into Powdery Product Containing Trehalose

DEPL:
Conversion into Powdery Product Containing Crystalline Trehalose

DEPL:
Conversion into Powder Product Containing Crystalline Trehalose

DEPL:
Conversion into Powdery Product Containing Anhydrous Crystalline Trehalose

DEPW:
Releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3;

DETL:
TABLE 1 Elution time Compo- Saccharide in on HPLC sition Substrate reaction mixture (min) (%) .alpha.-Glucosyltrehalose Trehalose 27.4 7.2 Glucose 33.8 3.9 .alpha.-Glucosyltrehalose 23.3 88.9 .alpha.-Maltosyltrehalose Trehalose 27.4 40.2 Maltose 28.7 40.5 .alpha.-Maltosyltrehalose 21.6 19.3 .alpha.-Maltotriosyltrehalose Trehalose 27.4 41.1 Maltotriose 25.9 58.2 .alpha.-Maltotriosyltrehalose 19.7 0.7 .alpha.-Maltotetraosyl- Trehalose 27.4 34.0 trehalose Maltotetraose 24.1 65.8 .alpha.-Maltotetraosyl- 18.7 0.2 trehalose .alpha.-Maltopentaosyl- Trehalose 27.4 29.1 trehalose Maltopentaose 22.6 70.6 .alpha.-Maltopentaosyl- 17.8 0.3 trehalose Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

CLPR:
3. The isolated DNA molecule according to claim 1, which is derivable from a microorganism of the genus Sulfolobus.

CLPR:
13. The isolated DNA molecule according to claim 10, which is derivable from a microorganism of the genus Sulfolobus.

CLPW:
Releasing trehalose from non-reducing saccharides having a trehalose structure

as an end unit and a degree of glucose polymerization of at least 3, but not substantially acting on maltooligosaccharides having a degree of glucose polymerization of at least 3;

CLPW:
Releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3, but not substantially acting on maltooligosaccharides having a degree of glucose polymerization of at least 3;

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	355	sulfolobus or acidocaldarius	USPAT; US-PGPUB	2002/06/05 15:10
2	L2	3835	trehalose	USPAT; US-PGPUB	2002/06/05 15:11
3	L4	110	non adj reducing adj saccharide$1	USPAT; US-PGPUB	2002/06/05 15:11
4	L6	3856	$trehalose	USPAT; US-PGPUB	2002/06/05 15:12
5	L7	31	1 and 6	USPAT; US-PGPUB	2002/06/05 15:12
6	L3	31	1 and 2	USPAT; US-PGPUB	2002/06/05 15:13
7	L5	19	1 and 4	USPAT; US-PGPUB	2002/06/05 15:29

US-PAT-NO: 6346394
DOCUMENT-IDENTIFIER: US 6346394 B1
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: February 12, 2002
US-CL-CURRENT: 435/69.1,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.2 ,435/71.1 ,435/71.2 ,435/97 ,536/23.1 ,536/23.2 ,536/23.7

APPL-NO: 9/ 055210
DATE FILED: April 6, 1998
PARENT-CASE:
CROSS-REFERENCE TO RELATED APPLICATIONS This is a continuation of application Ser. No. 08/798,269 filed Feb. 11, 1997, which is a division of application Ser. No. 08/505,377, filed Jul. 21, 1995, U.S. Pat. No. 5,856,146 the entire contents of both applications are herein incorporated by reference.

FOREIGN-APPL-PRIORITY-DATA:
FOREIGN-PRIORITY-APPL-NO: JP 6-190180
FOREIGN-PRIORITY-APPL-DATE: July 21, 1994

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	355	sulfolobus or acidocaldarius	USPAT; US-PGPUB	2002/06/05 15:10
2	L2	3835	trehalose	USPAT; US-PGPUB	2002/06/05 15:11
3	L4	110	non adj reducing adj saccharide$1	USPAT; US-PGPUB	2002/06/05 15:11
4	L6	3856	$trehalose	USPAT; US-PGPUB	2002/06/05 15:12
5	L7	31	1 and 6	USPAT; US-PGPUB	2002/06/05 15:12
6	L3	31	1 and 2	USPAT; US-PGPUB	2002/06/05 15:13
7	L5	19	1 and 4	USPAT; US-PGPUB	2002/06/05 15:29
8	L8	112	(2 or 4) near6 (synthes$8 or produc$8 or form$6) near6 enzym$8	USPAT; US-PGPUB	2002/06/05 15:39

PGPUB-DOCUMENT-NUMBER: 20020064816
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020064816 A1

TITLE: Moss genes from physcomitrella patens encoding proteins involved in the synthesis of carbohydrates

PUBLICATION-DATE: May 30, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Lerchl, Jens	Ladenburg		DE	
Renz, Andreas	Limburgerhof		DE	
Ehrhardt, Thomas	Speyer		DE	
Reindl, Andreas	Birkenheide		DE	
Cirpus, Petra	Mannheim		DE	
Bischoff, Friedrich	Mannheim		DE	
Frank, Markus	Ludwigshafen		DE	
Freund, Annette	Limburgerhof		DE	
Duwenig, Elke	Freiburg		DE	
Schmidt, Ralf-Michael	Kirrweiler		DE	
Reski, Ralf	Oberried		DE	

US-CL-CURRENT: 435/69.1,435/200 ,435/410 ,536/23.2

ABSTRACT:

Isolated nucleic acid molecules, designated CMRP nucleic acid molecules, which encode novel CMRPs from Physcomitrella patens are described. The invention also provides antisense nucleic acid molecules, recombinant expression vectors containing CMRP nucleic acid molecules, and host cells and organisms into which the expression vectors have been introduced. The invention still further provides isolated CMRPs, mutated CMRPs, fusion proteins, antigenic peptides and methods for the improvement of production of a desired compound from transformed cells based on genetic engineering of CMRP genes in this organism.

DATE FILED: December 13, 2000

---------- KWIC ---------

BSTX:
[0070] Trehalose-6-phosphate is formed from UDP-glucose and glucose-6-phosphate by the enzyme trehalose-6-phosphate synthase Trehalose-phosphate phosphatase than forms trehalose (Goddijn O. J. M. and van Dun, K. (1999) Trends in Plant Science 4: 315-319). Trehalose is cleaved into two glucose molecules by the enzyme alpha,alpha-Trehalase. Beside sucrose and trehalose, raffinose, stachyose and verbascose as well as sugar-alcohol's are important

transport-forms of carbohydrates (Zimmermann et al (1975) Encyclopedia of Plant Physiology, Vol I, Suringer Verlag Heidelberg: pp. 480-503). Raffinose is synthesized by the enzymes galactiol synthase and raffinose synthase. Raffinose and stachyose synthetic enzymes have been described from several plants (see e.g. Peterbauer, T. and Richter, A. (1998) Plant Physiol. 117: 165-172.

PGPUB-DOCUMENT-NUMBER: 20020058101
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020058101 A1

TITLE: CRYSTALLINE TREHALOSE DIHYDRATE, ITS PREPARATION AND USES

PUBLICATION-DATE: May 16, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
OHASHI, TETSUYA	OKAYAMA		JP	
CHAEN, HIROTO	OKAYAMA		JP	
MIYAKE, TOSHIO	OKAYAMA		JP	

US-CL-CURRENT: 426/658

ABSTRACT:

Disclosed are a crystalline trehalose dihydrate which has an elongated crystalline structure with a proportion of the length in the c axis to that in the b axis less than 2.0, its preparation and uses. The crystal has insubstantial hygroscopicity and solidification, and satisfactory stability, free-flowing ability, and handleability; it can be easily processed without fracture in the drying and sieving steps.

DATE FILED: July 8, 1999

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	DOC-ID	APPL-DATE
JP	194356/1998	1998JP-194356/1998	July 9, 1998

---------- KWIC ---------

BSTX:
[0005] Industrial-scale production of crystalline trehalose dihydrate has been realized, for example, by using in combination several enzymes, in particular, a non-reducing saccharide-forming enzyme, as disclosed in Japanese Patent Kokai No. 143,876/95, which forms a non-reducing saccharide having a trehalose structure as an end unit from one or more reducing partial starch hydrolysates with a glucose polymerization degree of at least three; and a trehalose-releasing enzyme, as disclosed in Japanese Patent Kokai No. 213,283/95, which specifically hydrolyzes the bonding between the part of a trehalose structure and the resting part of a non-reducing saccharide having a trehalose structure as an end unit and a glucose polymerization degree of at least three. Journal of Chemical Physics, Vol. 77, No. 5, pp. 2,330-2,335 (1982) reported the structure of crystalline trehalose dihydrate which has an orthorhombic structure as shown in FIG. 1, and has axes having different

lengths and crossings at right angles, i.e., it has the a, b, and c axes, and which more elongates to the direction of the c axis than to the b axis, resulting in growth of an easily fragile slender shape of crystal. In fact, commercially available crystalline trehalose dihydrate has a rather slender shape as shown in FIG. 2, a microscopic photograph, where the proportion of the length to the direction of the c axis (the length to the direction of the c axis is abbreviated as the c axis throughout the specification, unless specified otherwise) to that of the b axis (the length to the direction of the b axis is abbreviated as the b axis throughout the specification, unless specified otherwise) is about 3.5 to about 5.5 folds and the c axis is about 2 mm at the longest. Commercially available crystalline trehalose dihydrate has a proportion of length to the direction of the c axis to that of the a axis (the length to the direction of the a axis is abbreviated as the a axis throughout the specification, unless specified otherwise) is about 8 to about 12 folds. The aforesaid conventional crystalline trehalose dihydrate is generally too large in surface area, resulting in the following drawbacks: It is not easily separated from molasses in the separation steps; it needs a relatively-large amount of drying energy; and it is easily fragile during the steps of drying and sieving. Moreover, conventional slender shape of crystalline trehalose dihydrate could not hardly process candy fluff using commercially available machines therefor and hardly be used to process alcoholic beverages with fruits such as ume brandy because the crystal easily dissolves in water. Unlike conventional crystalline trehalose dihydrate, greatly expected is the establishment of a crystalline trehalose dihydrate that can be easily separated and dried in its processing, free of fracture during the steps of drying and sieving, and arbitrarily used to produce candy fluffs and alcoholic beverages with fruits.

PGPUB-DOCUMENT-NUMBER: 20020055620
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020055620 A1

TITLE: Reduction inhibitory agent for active oxygen eliminating activity

PUBLICATION-DATE: May 9, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Aga, Hajime	Okayama		JP	
Shibuya, Takashi	Okayama		JP	
Fukuda, Shigeharu	Okayama		JP	
Miyake, Toshio	Okayama		JP	

US-CL-CURRENT: 536/4.1

ABSTRACT:

A reduction inhibitory agent for active oxygen eliminating activity comprises trehalose as an active ingredient. This agent can be incorporated into plant edible products and/or plant antioxidants.

DATE FILED: October 30, 2001

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	DOC-ID	APPL-DATE
JP	63987/1997	1997JP-63987/1997	March 4, 1997
JP	17647/1998	1998JP-17647/1998	January 14, 1998

---------- KWIC ---------

DETX:
[0049] Corn starch was prepared into an about 33% starch suspension which was then mixed with 0.1% calcium carbonate, adjusted to pH 6.5, mixed with 0.3 per g starch of "TERMAMYL", an .alpha.-amylase specimen commercialized by Novo Industri A/S Copenhagen, Denmark, enzymatically reacted at 95.degree. C. for 15 min, autoclaved at 120.degree. C., and promptly cooled to obtain a liquefied solution with DE of about four. Thereafter, the liquefied solution was admixed with four units/g starch, d.s.b., of respective non-reducing saccharide-forming enzyme and trehalose-releasing enzyme, prepared by culturing a microorganism of the genus Rhizobium by the method in Japanese Patent Kokai No. 213,283/93, 500 units/g starch of isomaylase, and five units/g starch of cyclomaltodextrin glucanotransferase, adjusted to pH 6.2, and enzymatically reacted at 40.degree. C. for 48 hours. The reaction mixture was heated to inactivate the remaining enzymes, then admixed with 10 unites/g substrate of

glucoamylase and enzymatically reacted at pH 5.0 and 50.degree. C. for 10 hours. The reaction mixture thus obtained contained about 86% trehalose, d.s.b. The mixture was heated to inactivate the remaining enzyme, then in a conventional manner purified by decoloring and desalting, and continuously crystallized while concentrating. The resulting massecuite was separated by a basket-type centrifuge, and the crystal was washed by spraying with a small amount of water to obtain a crystalline trehalose hydrate with a purity of 98% or higher in a yield of about 64%, d.s.b. The product is a crystalline trehalose hydrate with a considerably-high purity and suitably used as the present reduction inhibitory agent and advantageously used to inhibit the reduction of active-oxygen eliminating activity of plant edible substances.

DETX:

[0051] Potato starch was prepared into a 10% starch suspension which was then subjected to the action of .alpha.-amylase to obtain a liquefied solution. To the liquefied solution were added three units/g starch, d.s.b., or a non-reducing saccharide-forming enzyme disclosed in Japanese Patent Kokai No. 213,283/95, five units/g starch, d.s.b., of a trehalose-relaeasing enzyme, 1,000 units/g starch, d.s.b., of an isoamylase, and one unit/g starch, d.s.b., of a maltotetraose-forming amylase, and the mixture was enzymatically reacted at pH 6.0 and 40.degree. C. for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, and in a conventional manner purified by decoloring and desalting and concentrated in vacuo to obtain a syrup with a moisture content of about 30% and DE of about 15 in a yield of about 90%, d.s.b. The product is a stable syrup, which contains, on a dry solid basis, about 50% trehalose along with other saccharides derived from starch, can be suitably used as a reduction inhibitory agent for active-oxygen eliminating activity, and arbitrarily used to inhibit the reduction of active-oxygen eliminating activity of plant edible substances.

PGPUB-DOCUMENT-NUMBER: 20020055486
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020055486 A1

TITLE: Pharmaceutical composition for ophthalmic use

PUBLICATION-DATE: May 9, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Matsuo, Toshihiko	Okayama		JP	
Kurimoto, Masashi	Okayama		JP	
Yamauchi, Hiroshi	Okayama		JP	

US-CL-CURRENT: 514/53,514/400 ,514/419 ,514/423 ,514/561

ABSTRACT:

An ophthalmic pharmaceutical composition comprising trehalose as an effective ingredient and a pharmaceutically-acceptable carrier. The pharmaceutical composition is a safe, long-term continuously-administrabl- e, therapeutic and/or prophylactic agent for the ophthalmologic clinical symptoms and signs in Sjogren syndrome.

DATE FILED: August 30, 2001

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	DOC-ID	APPL-DATE
JP	280023/2000	2000JP-280023/2000	September 14, 2000

---------- KWIC ---------

BSTX:
[0010] Any trehalose can be used in the present invention independently of its preparation methods and origin, as long as it does not spoil the present object. Examples of the methods for producing .alpha.,.alpha.-trehalose include the enzymatic methods as disclosed in Japanese Patent Kokai Nos. 143,876/95, 213,283/95, 322,883/95, 298,880/95, 66,187/96, 66,188/96, 336,388/96, and 84,586/96, where non-reducing saccharide-forming enzymes and trehalose-releasing enzymes are allowed to act on starch hydrolysates to form .alpha.,.alpha.-trehalo- se. The trehalose thus obtained can be advantageously used because of its economical merit and lesser possibility of the contamination of harmful impurities as compared with those prepared by synthetic methods. Examples of commercialized trehalose produced by the above enzymatic methods are "TREHA.RTM.", a trehalose having a trehalose content of 98%, on a dry solid basis (d.s.b.), commercialized by Hayashibara Shoji, Inc., Okayama, Japan; and a reagent grade trehalose having a trehalose content of 99%

or higher, d.s.b., commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. The trehalose usable in the present invention should not be restricted to the ones above, however, the above trehalose products can be advantageously used.

PGPUB-DOCUMENT-NUMBER: 20020042393
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020042393 A1

TITLE: Excipient

PUBLICATION-DATE: April 11, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Oobae, Kazuhiro	Nobeoka-shi		JP	
Kamada, Etsuo	Nobeoka-shi		JP	
Gomi, Shun?apos; ichi	Nobeoka-shi		JP	

US-CL-CURRENT: 514/53

ABSTRACT:

An excipient comprising trehalose having a purity of 99.0% or more, a proportion of particles of 75 .mu.m or more of 2 to 90 wt %, an average particle size of 10 to 250 .mu.m, an apparent specific volume of 1.5 to 3.5 ml/g, and a whiteness of 90% or more.

DATE FILED: January 12, 2001

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	DOC-ID	APPL-DATE
JP	10-200295	1998JP-10-200295	July 15, 1998
JP	10-323560	1998JP-10-323560	November 13, 1998

---------- KWIC ---------

BSTX:
[0036] The trehalose referred to herein is that obtained by treating one or more starch degradation products having a degree of glucose polymerization of 3 or more, with enzymes. From the viewpoint of cost, it is preferable for industrial utilization to use trehalose obtained by purification, grinding, particle size adjustment and the like of the following starting material: for example, commercial trehalose ("Trehaose" available from Hayashibara Biochemical Laboratories, Inc.) or the trehalose disclosed in JP-A-7-143876 and produced from one or more starch degradation products, for example, by a process using enzymes (a trehalose preparation having an increased trehalose content which is obtained by treating a solution containing one or more reducing starch partial-degradation products selected from those having a degree of glucose polymerization of 3 or more, with an enzyme capable of producing nonreducing sugars having a trehalose structure at the end from the

one or more reducing starch partial-degradation products selected from those having a degree of glucose polymerization of 3 or more, and then with glucoamylase or .alpha.-glucosidase to obtain a solution containing trehalose and sugars as contaminants, and subjecting this solution to a column chromatography using a strongly acidic cation-exchange resin.

PGPUB-DOCUMENT-NUMBER: 20020037321
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020037321 A1

TITLE: Instant water dissolvable encapsulate and process

PUBLICATION-DATE: March 28, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Vaghefi, Farid	Exton	PA	US	

US-CL-CURRENT: 424/494,264/4.1

ABSTRACT:

A microcapsule capable of thoroughly encapsulating environmentally-sensiti- ve or volatile core materials and capable of releasing said core material on contact with water. A process for manufacture of water soluble microcapsules comprising the admixture of a water soluble cellulosic material, a water soluble glucopyranosidyl material, at least two surfactants and core material, subjecting said mixture to an abrupt pressure change and drying the pressure-treated mixture.

DATE FILED: September 26, 2001

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DETX:
[0050] The most preferred glucopyranosidyl materials are 1-polyhydroxyalkylethers of glucopyranoside that may be prepared by the selective Raney nickel hydrogenation of the corresponding reducing disaccharides, which comprise all the naturally occurring disaccharides excluding sucrose and trehalose, and include the enzymatically rearranged products of the non-reducing disaccharides such as sucrose and trehalose. A special embodiment of this invention utilizes as the glucopyranosidyl material, an equimolar mixture of alpha-D-glucopyranosyl-1,1-D-mannitol and alpha-D-glucopyranosyl-1,6-D-sorbital, sold under the tradename Isomalt.

US-PAT-NO: 6391595
DOCUMENT-IDENTIFIER: US 6391595 B1
TITLE: Transferase and amylase, process for producing the enzymes, use thereof, and gene coding for the same
DATE-ISSUED: May 21, 2002
US-CL-CURRENT: 435/100,435/183 ,435/194 ,435/200 ,435/91.53 ,435/97

APPL-NO: 9/ 298924
DATE FILED: April 26, 1999
PARENT-CASE:
This application is a Divisional of application Ser. No. 08/750,569, filed Feb. 24, 1997, which is a national stage of PCT/JP95/01189 filed Jun. 14, 1995.

FOREIGN-APPL-PRIORITY-DATA:
FOREIGN-PRIORITY-APPL-NO: JP 6-133354
FOREIGN-PRIORITY-APPL-DATE: June 15, 1994

US-PAT-NO: 6372437
DOCUMENT-IDENTIFIER: US 6372437 B1
TITLE: Method for improving heat stability of RNA
DATE-ISSUED: April 16, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Hayashizaki; Yoshihide	Ibaraki	N/A	N/A	JPX

US-CL-CURRENT: 435/6,435/91.1 ,435/91.2 ,536/23.1 ,536/24.33 ,536/25.3
ABSTRACT:

A method for preparing a CDNA from a mRNA using a reverse transcriptase wherein reverse transcription is performed at a temperature at which the mRNA does not take a secondary structure, for example, at a temperature of 45.degree. C. or more. The method is performed, for example, using a heat-labile reverse transcriptase in the presence of a substance exhibiting chaperone function having chaperone function such as saccharides. The method is performed, for example, in the presence of metal ions necessary for activation of the reverse transcriptase and a chelating agent for the metal ions such as a deoxynucleotide triphosphate. The method is capable of reverse transcription over the full length of mRNA template even if the mRNA is a long chain mRNA and, as a result, producing a full length cDNA.

6 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

DATE FILED: March 13, 2001

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ORPL:

Carninci, P. et al., "Thermostabilization and Thermoactivation of Thermolabile Enzymes by Trehalose and its Application for the Synthesis of Full Length cDNA" Proc. Natl. Acad. Sci. USA, vol. 95, pp 520-524 (Jan., 1998).

US-PAT-NO: 6346394
DOCUMENT-IDENTIFIER: US 6346394 B1
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: February 12, 2002
US-CL-CURRENT: 435/69.1,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.2 ,435/71.1 ,435/71.2 ,435/97 ,536/23.1 ,536/23.2 ,536/23.7

APPL-NO: 9/ 055210
DATE FILED: April 6, 1998
PARENT-CASE:
CROSS-REFERENCE TO RELATED APPLICATIONS This is a continuation of application Ser. No. 08/798,269 filed Feb. 11, 1997, which is a division of application Ser. No. 08/505,377, filed Jul. 21, 1995, U.S. Pat. No. 5,856,146 the entire contents of both applications are herein incorporated by reference.

FOREIGN-APPL-PRIORITY-DATA:
FOREIGN-PRIORITY-APPL-NO: JP 6-190180
FOREIGN-PRIORITY-APPL-DATE: July 21, 1994

US-PAT-NO: 6323001
DOCUMENT-IDENTIFIER: US 6323001 B1
TITLE: Increasing the trehalose content of organisms by transforming them with combinations of the structural genes for trehalose synthase
DATE-ISSUED: November 27, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Londesborough; John	Helsinki	N/A	N/A	FIX
Tunnela; Outi	Espoo	N/A	N/A	FIX
Palva; Tapio	Uppsala	N/A	N/A	SEX
Holmstrom; Kjell-Ove	Uppsala	N/A	N/A	SEX
Welin; Bjorn	Uppsala	N/A	N/A	SEX
Mandal; Abul	Uppsala	N/A	N/A	SEX

US-CL-CURRENT: 435/69.1,435/320.1 ,435/419 ,435/468 ,536/23.2 ,536/23.7 ,536/23.74 ,536/24.1 ,800/295 ,800/298
ABSTRACT:
Two nucleotide sequences encoding two different polypeptides found in yeast trehalose synthase have been isolated and cloned. A third polypeptide has been isolated from the enzyme and characterized, and a method is provided to isolate and clone the nucleotide sequence encoding this polypeptide. The coding sequences can be inserted into suitable vectors and used to transform host cells. The transformed cells will produce increased amounts of trehalose compared to the untransformed wild types and have increased tolerance to a variety of stresses, in particular to decreased availability of water. The invention may be used to improve the stress tolerance of organisms, to increase the storage life of foodstuffs and to produce trehalose economically on an industrial scale in an organism (e.g. baker's yeast) that is a traditional and safe foodstuff.
27 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 29

DATE FILED: January 26, 1998

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DEPR:
These results disclose that the co-ordinate, 7-fold increase in Tre6P synthase and Tre6Pase activities that occurs during less than 2 h when glucose disappears from the medium is accompanied by increases in the amounts in yeast of three polypeptides, of mass 57, 99 and 123 kDa, that are immunoprecipitated by anti-TPS/P serum. These polypeptides are those found in the intact trehalose synthase purified in Example 1. Thus, increase in the amount of enzyme protein is a major mechanism by which the capacity of yeast to synthesize trehalose is increased.

DEPR:
Removal of the N-terminal 325 or so amino acids from the 123 kDa long chain of

intact trehalose synthase by treatment with trypsin in vitro produces an enzyme with catalytic properties like those of the truncated enzyme purified by Londesborough & Vuorio [(1991) loc. cit.]. In one experiment intact trehalose synthase (0.28 Tre6P synthase units, .apprxeq.9.4 .mu.g) was incubated with or without 0.5 .mu.g of trypsin at 30.degree. C. in 250 .mu.l of 13 mM HEPES pH 7.0 containing 1 mM MgCl.sub.2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.2 M NaCl and 0.5 mM benzamidine. Its Tre6P synthase activity was determined at intervals using standard assay mixtures (containing 5 mM F6P) containing no or 4 mM K phosphate pH 6.8, and samples were prepared for SDS-PAGE analysis immediately before and 48 min after addition of the trypsin.